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TYPE-1 RIBOSOME-INACTIVATING PROTEIN

Field of the Invention

The invention discloses a new type-1 ribosome-inactivating protein (RIP), referred to as bouganin, isolated from the leaves of *Bougainvillea* species, especially *B. spectabilis* Willd. Bouganin differs from other type-1 RIP by its unique amino acid composition. Bouganin has a molecular weight of about 26,200 daltons. Bouganin is useful as an immunomodulator, anti-viral agent or anti-tumour agent. Compositions comprising bouganin and a cell binding ligand are particularly useful to kill cells of a target population.

Background of the invention

Ribosome-inactivating proteins

It has been known for a long time that extracts from many plant tissues possess anti-viral activity, which in several cases is due to proteins identified as inhibitors of protein synthesis, called ribosome-inactivating proteins (RIP, reviewed by Barbieri *et al.*, *Biochim. Biophys. Acta* 1154:237 (1993)). The pokeweed anti-viral protein (PAP) was the first anti-viral protein to be identified as a RIP (reviewed by Irvin, in *Antiviral Proteins in Higher Plants* 65 (1994)). Subsequently, all other RIP tested possess anti-viral activity not only against plant viruses, but also against animal viruses, including HIV (reviewed by Battelli and Stirpe, in *Antiviral Proteins in Higher Plants* (1994)).

All RIP, either single-chain (type-1) or two-chain (type-2), enzymatically release adenine from a single nucleotide in a precise position (A_{4324} in the case of rat liver 28S rRNA, A_{2660} of *E. coli* rRNA) in a universally conserved GAGA tetraloop of the major rRNA (Endo and Tsurugi, *J. Biol. Chem.* 262:8128 (1987); reviewed by Barbieri *et al.*, *Biochim. Biophys. Acta* 1154:237 (1993)). Depurinated ribosomes become unable to elongate the nascent peptide chain.

The anti-viral activity of these proteins was commonly attributed to the inactivation of ribosomes, with inhibition of protein synthesis of the host cell and consequent arrest of viral replication. However a degradation of supercoiled DNA in the presence of RIP was reported (Li *et al.*, *Nucleic Acid Res.* 22:6309 (1991); Ling *et al.*, *FEBS Lett.* 345:143 (1994); Roncuzzi and Gasperi-Campani, *FEBS Lett.* 392:16 (1996)). Moreover, at least some RIP release more than one adenine residue from ribosomes

(Barbieri *et al.*, *Biochem. J.* 286:1 (1992)) and act on RNA species other than ribosomal, including viral RNAs, on poly(A), and on DNA (Barbieri *et al.*, *Nature* 372:624 (1994), *Nucleic Acid Res.* 25:518 (1997); Stirpe *et al.*, *FEBS Lett.* 382:309 (1996)). Thus many, if not all, RIP have polynucleotide:adenosine glycosidase activity, which may have a role in the anti-viral activity besides the inactivation of the host cell ribosomes.

Immunotoxins

Immunotoxins are chimeric molecules in which cell-binding ligands are coupled to toxins or their subunits. The ligand portion of the immunotoxin is usually an antibody that binds to selected target cells. The toxin portion of the immunotoxin can be derived form various sources. Most commonly, toxins are derived from plants or bacteria, but toxins of human origin or synthetic toxins (drugs) have been used as well. Toxins used for immunotoxins derived from plants or bacteria all inhibit protein synthesis of eukaryotic cells. The most widely used plant toxin, ricin, consist of two disulfide-linked polypeptides A and B (Olsnes *et al.*, in *Molecular Action of Toxins and Viruses* 51 (1982)). Another group of plant-derived toxins used in immunotoxins are the type-1 RIP. These molecules are single-chain proteins found in plants and have similar enzymatic properties as the A-chain of ricin (reviewed in Stirpe and Barbieri *FEBS Lett.* 195:1 (1986)).

The cross-linker used to join the ligand (antibody) and the toxin must remain stable when extracellular, but labile when intracellular, so that the toxin fragment can enter the cytosol. The choice of cross-linker depends on whether intact toxins, A-chains or type-1 RIP are used. A-chains and type-1 RIP are generally coupled to the ligand using linkers that introduce a disulfide bond between the ligand and the A-chain (Myers *et al.*, *J. Immunol. Meth.* 136:221 (1991)). Intact toxins are usually linked to ligands using non-reducible linkages (such as thioether) to prevent release of the active free toxin *in vivo*. Recombinant immunotoxins have been prepared by splicing the genes encoding the toxin to the gene encoding the ligand (for instance a recombinant antibody fragment) and expressing the entire immunotoxin as a fusion protein (Pastan *et al.*, *Ann. Rev. Biochem.* 61:331 (1992)). Recombinant immunotoxins are highly stable *in vivo* because they contain non-reducible peptide bonds.

Various types of immunotoxins directed against different cellular targets have been evaluated *in vivo*, both in animal models and in phase I or II clinical trials. The results of a number of these studies are reviewed in Ghetie and Vitetta *Curr. Opin. Immunol.* 6:707 (1994) and Thrush *et al.*, *Ann. Rev. Immunol.* 14:49 (1996).

Summary of the Invention

Ribosome inactivating proteins (RIP) comprise a class of proteins with potent inhibitory activity of eukaryotic protein synthesis. RIP can be classified in two groups. Type-1 RIP consist of a single peptide chain having ribosome inactivating activity, whereas type-2 RIP consist of an A chain with ribosome inactivating activity and a B chain having cell binding activity. Here we describe the isolation of a novel type-1 RIP, referred to as bouganin, with a low non-specific toxicity, making it very suitable for the incorporation as the toxin part in various immunotoxin molecules. The invention pertains to this novel protein and biologically active peptide parts and equivalents thereof, to immunotoxins based on this protein, to the production of such proteins and immunotoxins, and to their use in the medical and plant-protection fields. The invention is defined in the appending claims.

Detailed description of the Invention

The invention described herein draws on previously published work. By way of example, such work consists of scientific papers, patents and pending patent applications. All of these publications and applications, cited previously or below, are hereby incorporated by reference.

The protein according to the invention corresponds to the bouganin protein as described below in more detail, as well as to biologically active fragments and equivalents thereof. The term "biologically active" means being capable of inhibiting protein synthesis *in vitro* or *in vivo*. Such fragments generally comprise one or more active sites of the protein or the encoding polynucleotide and generally comprise a sequence at least 8 amino acids, preferably at least 10, at least 15 or even at least 30 amino acids of the protein, or the corresponding number of nucleotides of the polynucleotide.

The term "ligand" refers to any molecule capable of binding with or otherwise recognizing a receptor on a target cell. The ligand may be a protein or a non-protein molecule. Examples of such ligands include, but are not limited to, antibodies, growth factors, cytokines, hormones and the like, that specifically bind desired target cells.

As used herein, the term "immunotoxin" refers to chimeric molecules in which a cell binding ligand is coupled to the novel type-1 RIP bouganin or fragments thereof.

As used herein, the term "antibody" refers to polyclonal antibodies, monoclonal antibodies, humanized antibodies, single-chain antibodies, and fragments thereof such as

Fab, F(ab')2, Fv, and other fragments which retain the antigen binding function of the parent antibody.

As used herein, the term "monoclonal antibody" refers to an antibody composition having a homogeneous antibody population. The term is not limited regarding the species or source of the antibody, nor is it intended to be limited by the manner in which it is made. The term encompasses whole immunoglobulins as well as fragments such as Fab, F(ab')2, Fv, and others which retain the antigen binding function of the antibody. Monoclonal antibodies of any mammalian species can be used in this invention. In practice, however, the antibodies will typically be of rat or murine origin because of the availability of rat or murine cell lines for use in making the required hybrid cell lines or hybridomas to produce monoclonal antibodies.

As used herein, the term "humanized antibodies" means that at least a portion of the framework regions of an immunoglobulin are derived from human immunoglobulin sequences.

As used herein, the term "single chain antibodies" refer to antibodies prepared by determining the binding domains (both heavy and light chains) of a binding antibody, and supplying a linking moiety which permits preservation of the binding function. This forms, in essence, a radically abbreviated antibody, having only that part of the variable domain necessary for binding to the antigen. Determination and construction of single chain antibodies are described in U.S. Patent 4,946,778 to Ladner et al. Methods for the generation of antibodies suitable for use in the present invention are well known to those skilled in the art and can be found described in such publications as Harlow and Lane, *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory, (1988).

The natural or recombinant type-1 RIP bouganin molecules of the present invention may be fused to, or otherwise bound to a ligand by any method known and available to those skilled in the art. The ligand and the bouganin molecules may be chemically bonded together by any of a variety of well-known chemical procedures, such as the use of hetero-bifunctional cross-linkers, e.g. SPDP, 2-iminothiolane, carbodiimide or glutaraldehyde. Production of various immunotoxins is well-known within the art and can be found, for example in Thorpe *et al.*, *Monoclonal Antibody-Toxin Conjugates: Aiming the Magic Bullet* 168 (1982) and Waldmann, *Science*, 252:1657 (1991), both of which are incorporated by reference.

Bouganin may also be fused to the ligand by recombinant means such as through the production of single chain antibodies. The genes encoding ligand and bouganin may

be cloned in cDNA form and linked directly or separated by a small peptide linker by any cloning procedure known to those skilled in the art. See for example Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor laboratory, (1989).

A person skilled in the art will appreciate that additional modifications, deletions and insertions may be made to the ligand binding agent and bouganin genes. Especially, deletions or changes may be made in the linker connecting an antibody gene to bouganin. All such constructions may be made by methods of genetic engineering well known to those skilled in the art (see, generally, Sambrook et al., supra) and may produce proteins that have differing properties of affinity, specificity, stability and toxicity that make them particularly suitable for various clinical or biological applications.

Fusion proteins of the present invention including bouganin molecules may be expressed in a variety of host cells, including but not limited to bacterial hosts and yeast. The recombinant antibody–bouganin fusion protein gene will be linked to appropriate expression control sequences for each host. For E. coli this includes a promoter such as the T7, trp, or lambda promoters, a ribosome binding site and preferably a transcription termination signal. The plasmids of the invention can be transferred into the chosen host cell by well–known methods such as calcium chloride transformation. Cells transformed by the plasmids can be selected by resistance to antibiotics conferred by genes contained on the plasmids, such as, but not limited to, the ampiciline and neomycin genes.

Once expressed, the recombinant antibody–bouganin fusion proteins can be purified according to standard procedures of the art, such as described in R. Scopes, *Protein Purification*, Springer Verlag, N.Y. (1982). Substantially pure compositions of at least about 90 to 95% homogeneity are for pharmaceutical uses. Once purified, partially or to homogeneity as desired, the polypeptides may then be used therapeutically.

The pharmaceutical compositions of this invention are administered at a concentration that is therapeutically effective to a patient in the need of a treatment. To accomplish this goal, the pharmaceutical compositions may be formulated using a variety of acceptable excipients known in the art. The compositions for administration will commonly comprise a solution of the bouganin molecule, antibody–bouganin conjugates, single chain antibody–bouganin fusion proteins, ligand–bouganin conjugates or single chain ligand–bouganin fusion proteins dissolved in a pharmaceutically acceptable carrier, preferably an aqueous carrier. Typically, the compositions are administered by injection, either intravenously, intraperitoneally, in an other body cavity or into a lumen of an organ. Methods to accomplish this administration are known to those of ordinary skill in

the art. It may also be possible to obtain compositions which may be topically or orally administered, or which may be capable of transmission across mucous membranes.

The pharmaceutical compositions may also contain a polynucleotide encoding the bouganin molecule or active parts thereof, for use in gene therapy. The polynucleotide may be combined with means for specific delivery of the polynucleotide at the site were cells are to be killed by incorporation of the polynucleotide; such targeting means may e.g. be site-specific antibodies, liposomes or other art-known targeting devices.

Before administration to patients, formulants may be added to the antibodies. A liquid formulation is preferred. For example, these formulants may include oils, polymers, vitamins, carbohydrates, amino acids, salts, buffers, albumin, surfactants, or bulking agents. Preferably carbohydrates include sugar or sugar alcohols such as mono-, di-, or polysaccharides. The saccharides can include fructose, glucose, mannose, sorbose, xylose, lactose, maltose, sucrose, dextran, pullulan, dextrin, α- and β-cyclodextrin, soluble starch, hydroxyethyl starch, carboxymethyl cellulose, other water-soluble glucans, or mixtures thereof. Sucrose is most preferred. "Sugar alcohol" is defined as a C_4 to C_8 hydrocarbon having OH groups, and includes galactitol, inositol, mannitol, xylitol, sorbitol, glycerol, and arabitol. Mannitol is most preferred. These sugars or sugar alcohols mentioned above may be used individually or in combination. There is no fixed limit to the amount used, as long as the sugar or sugar alcohol is soluble in the aqueous preparation. Preferably, the sugar or sugar alcohol concentration is between 1.0 w/v% and 7.0 w/v%, more preferable between 2.0 and 6.0 w/v%. Preferably amino acids include levorotary (L) forms of carnitine, arginine, and betaine; however, other amino acids may be added. Preferred polymers include polyvinylpyrrolidone (PVP) with an average molecular weight between 2,000 and 3,000, or polyethylene glycol (PEG) with an average molecular weight between 3,000 and 5,000. It is also preferred to use a buffer in the composition to minimize pH changes in the solution before lyophilization or after reconstitution. Any physiologically acceptable buffer may be used, but citrate, phosphate, succinate, and glutamate buffers or mixtures thereof are preferred. Most preferred is a citrate buffer. Preferably, the concentration is from 0.01 to 0.3 M. Surfactants can be added to the formulation, for example those shown in EP-A-270799 and EP-A-268110.

Additionally, antibody-bouganin conjugates or single chain antibody-bouganin fusion proteins can, for example, be chemically modified by covalent conjugation to a polymer to increase their circulating half-life. Preferred polymers, and methods to attach them to peptides, are shown in U.S. Patents 4,766,106, 4,179,337, 4,495,285 and

4,609,546, which are all hereby incorporated by reference in their entireties. Preferred polymers are polyoxyethylated polyols and polyethylene glycol (PEG). PEG is soluble in water at room temperature and has the general formula: $R(O-CH_2-CH_2)_nO-R$ where R can be hydrogen, or a protective group such as an alkyl or alkanol group. Preferably, the protective group has between 1 and 8 carbons, more preferably it is methyl. The symbol n is a positive integer, preferably between 1 and 1,000, more preferably between 2 and 500. The PEG has a preferred average molecular weight between 1000 and 40,000, more preferably between 2000 and 20,000, most preferably between 3,000 and 12,000. Preferably, PEG has at least one hydroxy group, more preferably it is a terminal hydroxy group. It is this hydroxy group which is preferably activated to react with a free amino group on the inhibitor. However, it will be understood that the type and amount of the reactive groups may be varied to achieve a covalently conjugated PEG/antibody of the present invention.

Water soluble polyoxyethylated polyols are also useful in the present invention. They include polyoxyethylated sorbitol, polyoxyethylated glucose, polyoxyethylated glycerol (POG), etc. POG is preferred. One reason is because the glycerol backbone of polyoxyethylated glycerol is the same backbone occurring naturally in, for example, animals and humans in mono-, di-, triglycerides. Therefore, this branching would not necessarily be seen as a foreign agent in the body. The POG has a preferred molecular weight in the same range as PEG. The structure for POG is shown in Knauf *et al.*, *J. Biol. Chem.* 263:15064 (1988), and a discussion of POG/IL-2 conjugates is found in U.S. Patent 4,766,106, both of which are hereby incorporated by reference in their entireties.

Another drug delivery system for increasing circulatory half-life is the liposome. Methods of preparing liposome delivery systems are discussed in Gabizon *et al.*, *Cancer Res.* 42:4734 (1982); Cafiso, *Biochim. Biophys. Acta* 649:129 (1981); and Szoka, *Ann. Rev. Biophys. Eng.* 9:467 (1980). Other drug delivery systems are known in the art and are described in , e.g., Poznansky *et al.*, *Drug Delivery Systems* 253 (1980); Poznansky, *Pharm. Rev.* 36:277 (1984).

After the liquid pharmaceutical composition is prepared, it is preferably lyophilized to prevent degradation and to preserve sterility. Methods for lyophilizing liquid compositions are known to those of ordinary skill in the art. Just prior to use, the composition may be reconstituted with a sterile diluent (Ringer's solution, distilled water, or sterile saline, for example) which may include additional ingredients. Upon reconstitution, the composition is preferably administered to subjects using those methods

that are known to those skilled in the art. As stated above, the compositions of this invention are especially used to treat human patients. The preferred route of administraxtrose in saline. The vehicle may contain minor amounts of additives such as substances that enhance isotonicity and chemical stability, including buffers and preservatives.

The dosage and mode of administration will depend on the individual. Generally, the compositions are administered so that antibodies are given at a dose between 1 µg/kg and 20 mg/kg, more preferably between 20 µg/kg and 10 mg/kg. Preferably, it is given as a bolus. Continuous infusion may also be used, if so, the compositions may be infused at a dose between 1 and 100 µg/kg/min.

The compositions containing the present pharmaceutical compositions or a cocktail thereof (i.e., with other pharmaceutically active proteins) can be administered for therapeutic treatments. In therapeutic applications, compositions are administered to a patient suffering from a disease, in an amount sufficient to cure or at least partially arrest the disease and its complications. An amount adequate to accomplish this is defined as a "therapeutically effective dose." Amounts effective for this use will depend upon the severity of the disease and the general state of the patient's health.

Single or multiple administrations of the compositions may be administered depending on the dosage and frequency as required and tolerated by the patient. In any event, the composition should provide a sufficient quantity of the proteins of this invention to effectively treat the patient.

Among various uses of the pharmaceutical compositions of the present invention are included a variety of disease conditions caused by specific human cells that may be eliminated by the toxic action of the protein. One preferred application is the treatment of cancer, such as by the use of a tumour cell binding antibody as the ligand or of autoimmune conditions such as graft–versus–host disease, organ transplant rejection, type I diabetes, multiple sclerosis, rheumatoid arthritis, systemic lupus erythematosus, myasthenia gravis and the like. The pharmaceutical compositions may also be used *in vitro*, for example, in the elimination of harmful cells from bone marrow before transplant. The ligand portion of bouganin containing conjugates and fusion proteins is chosen according to the intended use. A large number of cell membrane molecules on lymphocytes may serve as target of the ligand part of the immunotoxin. Also antigens found on cancer cells that may serve as targets for the ligand part of immunotoxin with bouganin. Those skilled in the art will understand that ligands may be chosen that bind

to receptors expressed on other types of cells as well.

The bouganin molecule itself has also applications as an anti-viral compound. Type-1 RIP are know to be active against viruses affecting mammals and plants. Bouganin can therefore be used as a therapeutic molecule to treat viruses. The discovery of the anti-viral activity of RIP against a broad range of plant viruses when applied exogenous to inoculated leaves, has led to transfection of genes coding for RIP in host plants. Virus infection modifies the permeability of the cell membrane, thereby allowing the access of normally excluded molecules to the cytoplasm. RIP can then enter the virus infected cell and, once inside, inactivate ribosomes and viral replication. Besides the anti-viral activity of RIP, transfection of genes coding for RIP in host plant can also be applied to insect pest control. RIP are only moderately inhibitory for plant ribosomes but are highly inhibitory for ribosomes of plant parasites and are consequently good candidates for parasite control in plants. Transformation of an economically important host plant with the gene for a RIP which is toxic to parasites and is ineffective on the ribosomes of the plant confers specific resistance. An example of such a transgenic plant is a tobacco plant transfected with the Barley RIP. The constitutive expression of RIP in host plant can cause abnormal development of transgenic plant that can limit their application. To circumvent this problem a virus induced expression of RIP in transgenic plant is used, affecting only virus-infected cells without causing abnormal developing plants. Purified bouganin can also be applied directly in small amount on the leaves, completely preventing the mechanical transmission of unrelated viruses to several different host plants (Chen *et al.*, *Plant Pathol.* 40:612 (1991)).

The present invention will now be illustrated by reference to the following examples which set forth particularly advantageous embodiments. However, it should be noted that these embodiments are illustrative and are not to be construed as restricting the invention in any way.

Description of the Figures

Figure 1 shows the elution profile of this column step (solid line) in relation to the activity in the rabbit reticulocyte lysate assay (open circles). It can be seen that the activity in the rabbit reticulocyte lysate assay was resolved in several protein peaks.

Figure 2 shows the specificity of the polyclonal anti-bouganin serum in an ELISA experiment when bouganin was coated to the plates

Figure 3 shows a comparison of the N-terminal amino acid sequence of bouganin with

a number of other type-1 RIP. Amino acids are denoted by the single letter code.

Figure 4 shows the toxic activity of the bouganin immunotoxins based on anti-CD80 and anti-CD86 monoclonal antibodies (Mabs) when tested on CD80 and CD86 positive Raji cells. Toxic activity was evaluated from the inhibition of protein synthesis by the Raji cells.

Figure 5 shows the toxic activity of the bouganin immunotoxins based on anti-CD80 and anti-CD86 monoclonal antibodies (Mabs) when tested on CD80 and CD86 positive L428 cells. Toxic activity was evaluated from the inhibition of protein synthesis by the L428 cells.

Figure 6 shows the clonogenicity of $CD34^+$ staminal blood cells after short term exposure to the immunotoxins.

Figure 7 shows the clonogenicity of the Raji and L248 cell lines after short term exposure to the immunotoxins.

EXAMPLES

Example 1

Purification of bouganin, a novel type-1 RIP from the leaves of Bougainvillea spectabilis Willd

For the purification of the novel type-1 RIP, the following purification scheme was used. During the purification procedure, RIP activity was monitored using a rabbit reticulocyte lysate assay as described (Parente *et al.*, *Biochim. Biophys. Acta* 1216:43 (1993)). Reaction mixtures contained 10 mM Tris/HCl buffer, pH 7.4, 100 mM ammonium acetate, 2 mM magnesium acetate, 1 mM ATP, 0.2 mM GTP, 15 mM phosphocreatine, 3 μg of creatine kinase, 0.05 mM amino acids (minus leucine), 89 nCi of L-[^{14}C]-leucine, and 25 μl of rabbit reticulocyte lysate in a final volume of 62.5 μl. Incubation was at 28°C for 5 min. Protein concentration in the different purification steps was determined by spectrophotometry (Kalb *et al. Anal. Biochem.* 82:362 (1977)).

B. spectabilis leaves were obtained from the Botanic Garden of the University of Bologna (Italy). *B. spectabilis* leaves (1,400 gram) were ground in a mortar with liquid nitrogen and homogenized with an Ultraturrax apparatus in PBS (4 ml/g leaves). The slurry was extracted overnight at 4°C with magnetic stirring, filtered through cheesecloth, adjusted to pH 4.0 with glacial acetic acid, and centrifuged (10,000 x g) for 30 min. at 4°C. The acidified extract was applied to an S-Sepharose Fast Flow column (12 x 18 cm)

equilibrated with 10 mM sodium acetate, pH 4.5. The column was extensively washed with 5 mM sodium phosphate buffer, pH 7.0, and bound protein was eluted with 1 M NaCl in the same buffer. Active fractions were pooled and protein was precipitated by the addition of ammonium sulfate to saturation at 4°C. The precipitated material was recovered by centrifugation (10,000 x g) for 30 min. at 4°C. The pellet was dissolved and dialysed against water at 4°C, then clarified by centrifugation at (10,000 x g) for 30 min. at 4°C. The supernatant was adjusted to 5 mM phosphate buffer, pH 7.5, and applied to a CM-Sepharose Fast Flow column (30 x 1.6 cm) in the same buffer. The column was washed with the equilibration buffer and eluted with a NaCl linear gradient (from 0 to 200 mM in the same buffer, total volume 800 ml). Figure 1 shows the elution profile of this column step (solid line) in relation to the activity in the rabbit reticulocyte lysate assay (open circles). It can be seen that the activity in the rabbit reticulocyte lysate assay was resolved in several protein peaks. The protein peak denoted in Figure 1 as Peak 1 was analysed using reverse phase HPLC on a Vidac C4 column as described previously (Parente *et al.*, *Biochim. Biophys. Acta* 1216:43 (1993)) and gave only one single peak. It was therefore concluded that the activity was from a single protein. Table 1 summarizes the results of all the purification steps.

Table 1

Purification of RIP from leaves of *Bougainvillea spectabilis* Willd[a]

Preparation	Total protein (mg)	IC_{50} activity[b] (ng/ml)	Specific activity[c] (10^3 U/mg)	Total activity (10^6 U)	Yield (%)
acidified extract	3454	871	1.15	3.97	100
S-Sepharose eluate	300	100	10	3	75
CM-sepharose eluate					
- peak-1	3.5	10.5	95.5	0.33	8
- other active peaks	26.3	-	-	0.99	25

[a] results refer to 100 g of starting material
[b] IC_{50} is the protein amount that inhibits synthesis by 50% in a rabbit reticulocyte lysate system
[c] One unit (U) is the protein amount causing 50% inhibition of cell-free protein synthesis in 1 ml

Example 2

Characterization of bouganin

The purified protein peak 1 of example 1 was subjected to SDS-PAGE gel electrophoresis and analysed with a Epson GT8000 densitometer, utilizing a Gel Image

program (Pharmacia, Sweden). This analysis showed a single band of 26.2 kDa. The pI of the purified protein peak 1 was 9.0 (determined with a Phast System (Pharmacia) with the gels provided by the manufacturer). The absorption of the purified RIP from peak 1 was 8.72 (absorption was determined with water solutions of freeze-dried samples). The purified RIP from protein peak 1 is referred to as bouganin.

In order to obtain a polyclonal anti-bouganin serum for detection purposes, the isolated protein has been used for immunization of rabbits. The animals were immunized with 250 µg of isolated protein in a total volume of 1 ml containing 0.5 ml of the protein dissolved in PBS and 0.5 ml of complete Freund's adjuvant, by multi-site intradermic administration on day 0. Subsequent booster injections, also by multi-site intradermic administration, were given at day 14, 28 and 56 with the same amount of bouganin but now using Freund's incomplete adjuvant. Preimmune serum was taken followed by test bleedings on day 38 and 66. Animal were sacrificed on day 80 and a large batch of polyclonal anti-bouganin serum was obtained. The polyclonal anti-bouganin serum is able to bind specifically to bouganin in ELISA (Figure 2), when bouganin was coated to the ELISA plates, and in Western blot analysis.

Example 3

Partial amino acid sequencing of bouganin

The N-terminal amino acid sequence of bouganin was determined by the method described by Parente *et al.*, *Biochim. Biophys. Acta* 1216:43 (1993). The N-terminal amino acid sequence of bouganin is shown below.

Bouganin (SEQ ID NO:1)

Y N T V S F N L G E A Y E Y P T F I Q D L R N E L A K G T P

The N-terminal amino acid sequence of bouganin was compared to know protein sequences using the BLAST search method of the National Center for Biotechnology Information (NCBI) (Altschul *et al.*, *J. Mol. Biol.* 215:403 (1990)). This protein data base search revealed that bouganin does not match with any known protein sequence. It can be seen in Figure 3 that bouganin has only limited homology to other known type-1 RIP. The amino acid identity of bouganin with known type-1 RIP ranged from 20% to 37% and was clearly confined to a number of conserved amino acid residues.

Internal amino acid sequence data were obtained by digesting the isolated bouganin protein using V8 protease. The proteolytic generated peptide fragments were analysed using SDS-PAGE electroforesis and subsequently electroblotted to a poly-

vinylidene difluoride (PVDF) membrane. Using Edman Degradation for amino acid sequencing, one internal amino acid sequence was revealed. This sequence is as follows:
Bouganin (SEQ ID NO:2):
(E) L G V Y K L E F S I E A I (W) G K T Q N G
The amino acids placed between brackets in the obtained sequence are uncertain.

Example 4

Biological characterization of bouganin

From Table 1 above, it was calculated that bouganin inhibits protein synthesis in the rabbit reticulocyte lysate assay with an IC_{50} of 4.01×10^{-11}M. Bouganin was also tested for the inhibition of protein synthesis of various human cell lines. The cell lines used, namely mouse 3T3(fibroblasts), and human HeLa (carcinoma), NB100 (neuroblastoma) and BeWo (chorion carcinoma) were maintained as monolayer cultures in RPMI 1640 medium supplemented with antibiotics and 10% fetal calf serum, in a humidified atmosphere containing 5% CO_2, at 37°C. Subcultures were obtained by trypsin treatment of confluent cultures. The human JM cell line (monocyte-derived) was grown in suspension and treated with phorbol myristate acetate to induce adhesion as described (Bolognesi *et al.*, *Eur. J. Biochem.* 228:935 (1995)). Protein synthesis by various cell lines was assayed as described previously (Ferreras *et al.*, *Biochim. Biophys. Acta* 1216: 31 (1993)). Cells (10^5/well) were incubated with bouganin for 18 h., followed by a 2 h. pulse with L-[4,5-^{3}H]leucine (125 nCi/0.25 ml, obtained from Amersham International, Bucks., UK). The IC_{50} (concentration giving 50% inhibition) was calculated by linear regression analysis. Table 2 shows that the bouganin concentrations needed to inhibit protein synthesis of these human cell lines were much higher than the concentration needed to inhibit the protein synthesis in the rabbit reticulocyte lysate assay. This indicates that the cells tested do not have specific receptors to internalize bouganin.

Bouganin was also tested for its capacity to release adenine from various sources. Poly(A) and rRNA from *Escherichia coli* (16S + 23S, m.wt. 1.75×10^6) were from Boehringer GmbH, Mannheim, DE. DNA from herring sperm (Sigma Chemical Co., St. Louis, MO, USA) was mechanically sheared and made RNA-free by treatment with DNase-free RNase A (Boehringer GmbH, Mannheim, DE) for 2.5 h. at 37°C. DNA was then repeatedly precipitated in ethanol to remove the enzyme. Genomic RNA (m ssRNA positive + one small satellite, m.wt. 1.49×10^6) from artichoke mottled crinkle virus (AMCV) was prepared by phenol extraction and ethanol precipitation from purified virus

isolates. Rat liver ribosomes were prepared essentially as described elsewhere (Arias *et al.*, *Planta* 186:532 (1992)) in RNase-free conditions. Their concentration was determined by the A_{260} according to Montanaro *et al. Biochem. J.* 176:371 (1978), assuming that 12.5 AU/ml were equivalent to 1 mg/ml and that 1 mg contained 250 pmol of ribosomes. Ribosomes were stored in aliquots at -80°C.

Table 2

Effect of *B. spectabilis* RIP on protein synthesis by cell lines[a]

Cell line	Origin	Incorporation of [^{3}H] leucine by control cells(dpm ± SD)	Inhibition of protein synthesis (IC_{50}[b])
JM	monocytes	8555 ± 824	1218 ± 484
HeLa	carcinoma	24082 ± 6367	>3300
NB100	neuroblastoma	12607 ± 3694	665 ± 0
BeWo	chorion carcinoma	18995 ± 7332	950 ± 16
3T3	fibroblasts	4317 ± 2652	>3300

[a] Results are mean values ± S. D. of two experiments performed in triplicate.

[b] IC_{50}: concentration of protein inhibiting protein by 50% as compared to controls.

Determination of polynucleotide:adenosine glycosidase activity was determined by measuring adenine (obtained from Sigma Chemical Co., St. Louis, MO, USA) released from the various sources by HPLC (Zamboni *et al.*, *Biochem. J.* 259:639 (1989)), essentially following the procedure of McCann *et al. Antimicrob. Agents Chemother.* 28:265 (1985) as described by Stirpe *et al. FEBS Lett.* 382:309 (1996). Reactions were run for 40 min. at 30°C in a final volume of 50 µl containing 50 mM sodium acetate, pH 4.0, 100 mM KCl, bouganin and substrate. Controls were run without bouganin, and a standard curve of adenine was run with each experiment. Bouganin not only released adenine from rat liver ribosomes (one mole of adenine per ribosome, approximately), but also from *E. coli* rRNA, from poly(A), from genomic AMCV RNA and from herring sperm DNA. Among polynucleotides, DNA appeared the best substrate. The number of adenine residues released was near to one per ribosome, and several per mol of rRNA or AMCV RNA.

Example 5

In vivo toxicity of bouganin

Bouganin was also tested for toxicity in animals. Various doses were injected i.p. to groups of three male and three female Swiss mice. The ratio between doses was two, and the animals were observed up to 16 days after treatment. Other known RIP have a toxicity (LD_{50} values) in the range of 1 to 40 mg/kg (Barbieri *et al., Biochim. Biophys. Acta* 1154:237 (1993)). Bouganin was not toxic in the test animals at a dose as high as 32 mg/kg.

Example 6

Generation of chemically coupled anti-CD80 and anti-CD86 immunotoxin molecules containing bouganin

Immunotoxins containing bouganin were prepared essentially according to the method described by Bolognesi *et al. Clin. Exp. Immunol.* 89:341 (1992). Anti-CD80 (Mab B7-24), anti- CD86 (Mab 1G10) and bouganin, the latter containing a trace of ^{125}I-RIP, were dissolved in 50 mM sodium borate buffer, pH 9.0, at a concentration of 1 mg/ml, 1.5 mg/ml and 2.5 mg/ml, respectively, and were modified by adding 2-imino-thiolane (Sigma) to a final concentration of 0.6 mM (Mab), 1.0 mM (bouganin). After 60 min. at room temperature (21°C), glycine was added to a final concentration of 200 mM, and after further 20 min. Ellman's reagent, dissolved in 50 Fl of dimethylformamide, was added to a final concentration of 2.5 mM. After 10 min. at room temperature the reaction mixture was filtered through a Sephadex G25 column, and the number of sulphydryl groups introduced was determined. The derivatised RIP were reduced with 20 mM 2-mercaptoethanol, filtered through a Sephadex G25 column, and were then mixed with the modified Mab in a RIP:Mab ratio 10:1. After 18 h. at room temperature the conjugates were separated from the unreacted reagents by gel filtration on a Sephacryl S200 high-resolution column, equilibrated and eluted with phosphate buffered saline (PBS, 0.14 M NaCl in 5 mM sodium phosphate buffer, pH 7.4). Protein synthesis inhibitory activity of the fractions was assayed on a rabbit reticulocyte lysate as described (Bolognesi *et al., Eur. J. Biochem.* 228:935 (1995)).

The RIP:Mab ratio in the conjugates was estimated from the ^{125}I-RIP radio-activity and from the protein concentration calculated from the A_{280}. The conjugates were sterilized by filtering through a 0.22 µm filter and stored at 4°C at concentration higher than 10^{-6} M.

Example 7

Characterization of chemically coupled anti-CD80 and anti-CD86 immunotoxin molecules containing bouganin

The activity of the bouganin containing conjugates in comparison to the activity of gelonin and saporin containing conjugates coupled to anti-CD80 and anti-CD86 Mabs, was assayed on the Raji cell line, derived from a Burkitt lymphoma, and the L428 cell line, derived from a Hodgkin's lymphoma. Cells were maintained in RPMI 1640 medium with 10% fetal bovine serum (FBS) (Gibco), glutamine (Sigma) and antibiotics (Bio-whittaker). The type 1 RIPs gelonin and saporin (saporin-S6), were purified as previously described by Barbieri *et al. J. Chromatography* 408:235 (1987) from the seeds of *Gelonium multiflorum* and *Saponaria officinalis*, respectively, subsequently anti-CD80 and anti-CD86 immunotoxins were prepared containing gelonin and saporin as was described above for the bouganin containing immunotoxins.

Before each series of experiments the reactivity of the target cells with anti-CD80 and anti-CD86 Mabs was ascertained by means of immunofluorescence and flow cytometry. Briefly, cells, harvested and checked for viability by trypan blue dye exclusion, were adjusted to a concentration of 10^6 cells/ml of complete RPMI 1640 medium. To 100 µl of cell suspension, 100 µl of a 10^{-7} M solution of the Mabs were added. Negative samples were run with appropriate isotype-matched irrelevant Mab. Cells were incubated for 30 min. at 4°C, washed twice in phosphate-buffered saline containing 1% FBS, and incubated again in a volume of 50 µl with 4 µl of FITC-GAM. After three washings with PBS containing 1% FBS the samples were fixed with PBS containing 1% formalin. Binding of Mabs was assessed by flow cytometry, with an EPICS XL equipment (Coulter). Histograms and statistics were generated with the software of the EPICS-dedicated computer. Both cell lines were found to be positive for expression of both CD80 and CD86.

The inhibitory activity of immunotoxins on cell-free protein synthesis was evaluated with a rabbit reticulocyte lysate. Immunotoxins were prior reduced with 20 mM 2-mercaptoethanol for 30 min. at 37°C, appropriatly diluted and then added to a reaction mixture containing, in a final volume of 62.5 µl: 10 mM Tris/HCl buffer, pH 7.4, 100 mM ammonium acetate, 2 mM magnesium acetate, 1 mM ATP, 0.2 mM GTP, 15 mM phosphocreatine, 3 µg of creatine kinase, 0.05 mM amino acids (minus leucine), 3.3 kBq of L-^{14}C-leucine (Amersham International, Bucks, UK) and 25 µl of a rabbit reticulocyte lysate. Incubation was at 28°C for 5 min. The reaction was arrested with 1 ml of 0.1 M

potassium hydroxide, and two drops of hydrogen peroxide and 1 ml of 20% (w/v) of trichloroacetic acid were added. Precipitated proteins were collected on glass-fibre discs and the radioactivity incorporated was measured with a β-counter (Beckman), after the addition of 5 ml of Ready Gel scintillation cocktail (Beckman) containing 0.7 % acetic acid. Each experiment was carried out in duplicate. The concentration of immunotoxins, expressed as RIP content, causing 50% inhibition of leucine incorporation (IC_{50}) was calculated by linear regression analysis. Table 3 shows the key characteristics of the immunotoxins used.

Table 3

Characteristics of the derivatized Mabs and RIPs and of the immunotoxins

	Mab		RIP			Immunotoxin*	
	2-IT (mM)	Thiol groups inserted (mol/mol)	2-IT (mM)	Thiol groups inserted (mol/mol)	IC_{50}* (ng/ml)	RIP/Mab (mol/mol)	IC_{50}* (ng/ml)
anti-CD80-bouganin	0.6	2.28	1.0	0.88	16.2	3.07	22.7
anti-CD80-gelonin	0.6	2.83	1.0	1.06	20.9	3.67	29.8
anti-CD80-saporin	0.6	2.54	1.0	1.41	2.6	2.11	7.6
anti-CD86-bouganin	0.6	1.28	1.0	0.65	16.2	2.66	27.7
anti-CD86-gelonin	0.6	3.01	1.0	0.74	20.9	2.73	50.1
anti-CD86-saporin	0.6	2.61	1.0	1.32	2.6	2.41	5.8

* expressed as concentration of the RIP.

Six different immunotoxins were obtained with the anti-CD80 and anti-CD86 monoclonal antibodies and three different single chain RIPs (bouganin, gelonin, and saporin). The RIPs were conjugated to the Mabs by an artificial disulphide bond. Sulphydryl groups were inserted in each type of molecule by an imidoester reaction between 2-iminothiolane and the primary amino-groups of the proteins. Both Mabs showed a marked reactivity with 2-iminothiolane, with an average of more than 2.5 SH groups inserted per molecule, using a standard concentration of the linking reagent. The three RIPs were less reactive, and amongst them bouganin showed the lower, and saporin the highest, derivatisation grade. After conjugation the toxin/Mab molar ratio resulted of about 2.5 for the anti-CD86 containing immunotoxins, whilst those containing the anti-CD80 Mab gave more variable products, with the toxin/Mab molar ratios ranging from 2.11 to 3.67. The inhibitory activity of native and conjugated RIPs on protein synthesis

by a rabbit reticulocytes lysate is also reported in Table 3. A loss of activity on conjugation was observed with all RIPs. This partial inactivation was minimal for saporin and was the greatest in the case of gelonin.

The cytotoxicity of the immunotoxins was evaluated from the inhibition of ^{3}H-leucine incorporation in CD80/86 positive cell lines. Raji and L428 cells were harvested, checked for viability and adjusted to a concentration of 10^5 cells/ml in complete RPMI 1640 medium. Cells (10^4) were seeded in 96-wells microtiter plates in a volume of 200 µl containing anti-CD80 immunotoxins, or anti-CD86 immunotoxins, or a mixture of the two immunotoxins in concentrations ranging from 10^{-13} M to 10^{-8} M, of the RIP. Control samples were run with the respective RIP alone, the Mabs alone or a mixture of the Mabs and the free RIPs. In these experiments Ber-H2/saporin and B-B10/saporin were used as irrelevant immunotoxins for Raji and L428 cells, respectively. After 72 h. 74 kBq of ^{3}H-leucine (Amersham) was added. After another 18 h. cells were harvested with an automatic cell harvester (Skatron Instruments, Lier, Norway) onto glass-fiber diskettes. The radioactivity incorporated was determined as described above. The T24 cells were trypsinized and seeded in 24 well plates (2×10^4 cells/well in 0.5 ml), and used as control cells being CD80 and CD86 negative. After 24 h. the medium was removed and changed with medium containing variuos concentrations of immunotoxins (from 10^{-11} to 10^{-8} M, of the RIP). After 48 h of incubation, L-[4,5-^{3}H]leucine (74 kBq) was added in 100 µl volume of RPMI, and after further 18 h cells were fixed by adding 1 ml of 20% trichloroacetic acid. After three washes with 5% trichloroacetic acid, cells were lysed with 250 µl of 0.1 M potassium hydroxide, for 10 min. at 37 °C. The radioactivity was measured as described above. Each experiment was run in triplicate. Results are expressed as the mean of three different experiments, with a SD ≤10 %. All tested immunotoxins inhibited ^{3}H-leucine incorporation by Raji and L428 cell lines (Figure 4 and 5). RIPs incremented their toxicity on Raji cells by 3-4 log upon conjugation with anti-CD86 Mab and by 4-5 log upon conjugation with anti-CD80 Mab (Table 4). On L428 cells the pattern of toxicity was the same, but the increase of RIPs cytotoxicity upon conjugation was 1 log lower than on Raji cells (Table 5). No toxicity was observed with free Mabs. The anti-CD80-saporin and anti-CD86-saporin immunotoxins were the most active on cell lines, with IC_{50}'s ranging from 2.5×10^{-13} M to 5.8×10^{-12} M. The immunotoxins made with bouganin and gelonin showed IC_{50}'s in the $1.3-1.9\times10^{-10}$ M range, when linked to anti-CD86, and in the $4.6\times10^{-12}-5.7\times10^{-11}$ M range, when conjugated to anti-CD80. The immunotoxins containing anti-CD80 Mab were more active than the corresponding anti-CD86 Mab containing ones, whilst the mixture of the

two type of immunotoxins showed an intermediate toxicity. Similar results were obtained using either bouganin, gelonin or saporin, and in both Raji and L428 cell lines. Toxicity of the free RIPs was clearly the highest for saporin, followed by gelonin. Bouganin was clearly the least toxic at both cell lines.

Table 4

Effect of immunotoxins on protein synthesis by Raji cell line.

	anti-CD80 immunotoxins	anti-CD86 immunotoxins	anti-CD80 + anti-CD86 immunotoxins	Free RIPs
	IC_{50} (pM)	IC_{50} (pM)	IC_{50} (pM)	IC_{50} (nM)
Bouganin	4.61 (r^2 = 0.99)	192 (r^2 = 1.00)	12.2 (r^2 = 1.00)	839 (r^2 = 0.99)
Gelonin	56.5 (r^2 = 1.00)	172 (r^2 = 0.97)	82.1 (r^2 = 0.97)	541 (r^2 = 0.99)
Saporin	0.253 (r^2 = 0.99)	2.67 (r^2 = 0.99)	1.10 (r^2 = 1.00)	23.6 (r^2 = 0.99)

Table 5

Effect of immunotoxins on protein synthesis by L428 cell line.

	anti-CD80 immunotoxins	anti-CD86 immunotoxins	anti-CD80 + anti-CD86 immunotoxins	Free RIPs
	IC_{50} (pM)	IC_{50} (pM)	IC_{50} (pM)	IC_{50} (nM)
Bouganin	27.8 (r^2 = 0.99)	129 (r^2 = 1.00)	29.9 (r^2 = 1.00)	49.8 (r^2 = 1.00)
Gelonin	17.8 (r^2 = 1.00)	160 (r^2 = 0.99)	31.8 (r^2 = 0.98)	11.4 (r^2 = 0.99)
Saporin	0.495 (r^2 = 1.00)	5.84 (r^2 = 0.99)	2.46 (r^2 = 1.00)	4.37 (r^2 = 0.98)

The immunotoxins were also tested for capacity to inhibit clonogenic efficiency. Normal peripheral blood cells were cultured in semisolid medium as previously described (Tazzari *et al*, *Brit. J. Haematology* 86:97 (1994)). Briefly, 5×10^3 cells were plated in duplicate in culture medium consisting of 1 ml of Iscove's modified Dulbecco's medium (IMDM), supplemented with 24% FBS, 0.8% BSA, 10^{-4} M 2-mercaptoethanol, 2 U of human recombinant erythropoietin (Dompè Biotec, Milan, IT) and 0.2 mM bovine haemin. To measure the optimum clonogenic efficiency, 10% (v/v) of a selected batch

of a phytohemoagglutinin-lymphocyte conditioned medium was added.

Methylcellulose final concentration was 1.1%. Granulocyte-macrophage colony-forming unit (CFU-GM), erythroid progenitors (BFU-E) and mixed colonies (CFU-GEMM) were scored after 14 days of incubation at 37°C in a fully humidified 5% CO_2 atmosphere. All cultures were performed in presence of 2 U/ml of erythropoietin. Anti-CD80-bouganin and anti-CD86-bouganin immunotoxins were added to the cultures (continuous exposure) at a final concentration of 10^{-8} M as RIP. To control samples the same concentration of Mabs alone or bouganin alone was added. Experiments were also performed by plating highly purified $CD34^+$ cells after 1 h. incubation with 10^{-7} M immunotoxins, Mabs or bouganin (short-term exposure). The clonogenic efficiency of $CD34^+$ cells was 7 ± 3 %. $CD34^+$ cells were purified from the peripheral blood mononuclear fraction, obtained by gradient separation (Lymphoprep, 1077 g/l, Nycomed Pharma, Oslo, Norway). Low density cells were washed twice in phosphate buffer-saline with 1% bovine serum albumin (BSA, Sigma) and $CD34^+$ cells were highly purified by MiniMacs high-gradient magnetic separation column (Milteny Biotec, Bergisch Gladbach, DE) (Lemoli *et al.*, 1997). To assess the percentage of $CD34^+$ elements, aliquots of $CD34^+$ target cells were restained with the HPCA-2 antibody (IgG_{1a}-FITC, Becton Dickinson) directed toward an epitope of CD34 antigen different from the one targeted by the Qbend10 mAb, used with the MiniMacs system. Briefly, $CD34^+$ cells were incubated for 30 min. in the dark at 4°C with HPCA-2-FITC. Propidium iodide (2 µg/ml) was added for the detection of nonviable cells, which were excluded from analysis. After 2 washes in PBS/BSA, flow-cytometric analysis was performed on a gated population set on scatter properties by using FACScan equipment (Becton Dickinson). A minimum of 10,000 events were collected in list mode on FACScan software. In all experiments the purity of $CD34^+$ cells was > 90% and the recovery > 80% (Lemoli *et al.*, 1997). A short term exposure (1 h.) to 10^{-7} M concentration of all the tested substances showed an inhibition of CFU-mix, CFU-GM and BFU-e ranging from 20 to 50 %. Continuous incubation (14 days) with 10^{-8} M concentration of immunotoxins resulted in 52-71 % of inhibition, whereas a continuous exposure to the same concentration of free bouganin and Mabs gave 15-49 % inhibition (Figure 6).

The toxicity of short-term exposure to the bouganin-containing anti-CD80 and anti-CD86 immunotoxins was also tested on the clonogenic activity of L428 and Raji cell-lines. After 2 washes to remove free conjugates, 2×10^3 tumour cells were plated in IMDM supplemented with 10% FCS and 1% glutamine and antibiotics. Methylcellulose was added at a final concentration of 1.1%. Aggregates >50 cells were scored with an

inverted microscope after 7 days of culture. A complete elimination of L428 clones was reached with immunotoxins or cocktail treatment, whereas on Raji cells the anti-CD80 immunotoxin and the cocktail caused a total reduction of clonogenic growth, but the anti-CD86 did not achieve a complete elimination of clones. Free bouganin, anti-CD80 and anti-CD86 Mabs inhibited clonogenic growth from 0 to 22 % (Figure 7).

Example 8

Molecular cloning of the bouganin cDNA

The first step in the molecular cloning of the cDNA for bouganin was the design of degenerate PCR primers. These primers were based on the N-terminal amino acid sequence of bouganin or on the amino acid sequence of an internal peptide fragment of bouganin as are shown in example 3. Combinations of these primers or the individual primers together with an oligo-dT primer were used to amplify DNA fragments encoding bouganin. These fragments were sequenced in order to obtain cDNA sequence information.

Total RNA was isolated by pulverizing leaves of *B. spectabilis* Willd using liquid nitrogen and homogenizing in guanidine thiocyanate at 10 ml/g leaves. Next, the sample was extracted with phenol/chloroform/isoamyl alcohol, followed by precipitation of the RNA with ethanol. The RNA was washed with 75% ethanol and dissolved in DEPC-treated water. By measurement of the extinction at 260 nm the RNA was quantified. To obtain mRNA the sample was incubated with oligo-dT magnetic beads (Promega, Madison, USA). The mRNA was captured, eluted from the beads and quantified as specified by the manufacturers protocol. First strand cDNA was synthesized by incubation at 37°C for 1 h. of approximately 1µg mRNA or 10 µg0 in 50 µl mix, consisting of 1 x synthesis buffer (50 mM Tris-HCl pH 8.3, 75 mM KCl, 3 mM $MgCl_2$ and 10 mM DTT), 0.5 mM dNTP, random hexamers, M-MLV-reverse transcriptase (USB, Cleveland, Ohio, USA). From this mixture 1 - 2.5 µl was used as template in PCR reactions using the above described combinations of primers. A standard PCR mixture of 100 µl contained 1 x PCR buffer, 2.5 U Taq polymerase, 0.25 mM dNTPs, 250 nM of each primer and cDNA template. The mixture was run in a Perkin-Elmer thermocycler for 30 - 40 cycles of 1 min. 95°C, 1 min. 55°C - 57°C, and 2 min. 68°C - 72° C followed by 1 step for 7 min. at 68°C - 72°C as extension of the PCR product.

Based on the N-terminal amino acid sequence 4 sense and 1 anti-sense degenerate DNA primers were designed. Using appropriate pairs of primers various PCR

products were amplified. After analysis on ethidium bromide stained agarose gels it was initially observed that only the combination of primer 102 with 116 yielded a PCR product of expected size. The sequences of these degenerate primers are set out below using IUB nucleotide codons.

Primer 102 (SEQ ID NO: 3) 5' GGN GAR GCN TAY GAR TAY CCN AC 3'
Primer 116 (SEQ ID NO: 4) 5' GGN GTN CCY TTN GCN AGY TCR TT 3'

The 65 bp DNA fragment obtained in this way (corresponding to amino acid 10 to 30 of bouganin) was gel-purified and cloned in pCR-Script Cam Sk(+) cloning vector of Stratagene (La Jolla, USA) using the pCR-Script cloning kit according to the manufacturer's protocol. The DNA sequence of the insert was determined and the deduced amino acid sequence based on the resulting DNA sequence matched the experimentally determined N-terminal bouganin amino acid sequence. Below the retrieved sequence is shown.

Bouganin (SEQ ID NO: 5)
5' GGG GAG GCC TAC GAG TAT CCC ACT TTT ATA CAA GAT TTG CGC AAC GAA CTC GCT AAA GGA ACC CC 3'

Based on this sequence (SEQ ID NO: 5) the exact oligonucleotide primer 125 (SEQ ID NO: 6) was designed. This primer 125 was used in combination with the degenerate primer 197 (SEQ ID NO: 7), which was based upon the internal bouganin amino acid sequence obtained as described above in example 3. This PCR reaction resulted in a 360 bp fragment. The sequences of the used primers are set out below.

Primer 125 (SEQ ID NO: 6) 5' CTT TTA TAC AAG ATT TGC GCA ACG A 3'
Primer 197 (SEQ ID NO: 7) 5' AAY TCN ARY TTR TAN CAN CC 3'

The amplified 360 bp product was gel-purified and cloned in pCR-Script Cam Sk(+) cloning vector of Stratagene as described before. Subsequently, the DNA sequence was determined and the amino acid translation was deduced. The clone contains a fragment encoding 120 amino acids of bouganin (residues 17 – 136). The cDNA sequence and the amino acid sequence deduced from the sequence of this clone are shown in SEQ ID

No. 8. Also this deduced amino acid sequence shows limited identity with the amino acid sequences of several other known RIPs.

The partial amino acid sequences depicted in SEQ ID No.'s 1 and 2 (see Example 3) and the deduced partial amino acid sequence depicted in SEQ ID No. 8 were combined to the 149 amino acid sequence shown in SEQ ID No. 9, which represents about 60% of the complete bouganin amino acid sequence.

Example 9

Generation of single chain anti-CD86 immunotoxin molecules containing bouganin

A single-chain immunotoxin based on anti-CD86 monoclonal antibody and bouganin is obtained using a strategy by which a single chain antibody fragment (scFv) is transferred to an expression cassette system containing the pelB leader signal, the cDNA encoding bouganin and a 6 x his purification tag. In this expression plasmid, the scFv is cloned between the pelB leader signal and bouganin. The scFv-bouganin plasmid contains the Lac promoter that allows the expression of the immunotoxins after IPTG (isopropyl 8-D-thiogalactopyranoside) induction. BL21DE3 bacteria are transformed by the $CaCl_2$ method with the expression plasmid and plated on LB plates containing 100 µg/ml ampiciline. One colony is picked and grown overnight in LB containing 100 µg/ml ampicillin. Next day the culture is diluted (1/100) in LB containing 100 µg/ml ampiciline until the OD_{600} reaches ~0.5. At this point IPTG (Sigma Chemical Co. St. Louis, MO, USA)(0.1 - 1 mM) is added. After 3 h. the cells are harvested for purification of the recombinant scFv-immunotoxin. To purify the proteins from the periplasmic space, first the cells are harvested by centrifugation at 4000 x g for 20 min. and resuspended in 30 mM Tris/HCl, 20% sucrose, 0.5 mM EDTA, pH 8.0 and incubated on ice for 10 min. Subsequently the cells are centrifuged at 8000 x g for 20 min. and resuspended in ice cold 5 mM $MgSO_4$ followed by incubation on ice for 10 min. After centrifugation at 8000 x g the supernatant, which contains proteins from the periplasmic space, is collected and dialysed against 50 mM Na-phosphate, 300 mM NaCl, pH 8.0. This preparation is loaded on a Ni-NTA column (Qiagen, Chatsworth, USA), subsequently the column will be washed with 50 mM Na-phosphate, 300 mM NaCl, 10% glycerol, pH 6.0 and elution of the recombinant immunotoxins is done by 50 mM Na-phosphate, 300 mM NaCl, 10% glycerol, pH 4.0. Column fractions are analysed on SDS-PAGE; fractions containing immunotoxins are pooled and dialysed against suitable buffer.

SEQ ID NO:8

```
  1    T TTT ATA CAA GAT TTG CGC AAC GAA TTG GCT AAG GGC ACA CCA GTA   46
1 (17)   F   I   Q   D   L   R   N   E   L   A   K   G   T   P   V   15

 47  TGT CAA CTT CCA GTG ACA CTA CAA ACC ATA GCC GAT GAC AAG CGA TTT   94
 16   C   Q   L   P   V   T   L   Q   T   I   A   D   D   K   R   F   31

 95  GTT CTA GTT GAT ATC ACT ACG ACC TCG AAG AAA ACA GTT AAG GTT GCT  142
 32   V   L   V   D   I   T   T   T   S   K   K   T   V   K   V   A   47

143  ATA GAT GTG ACA GAT GTG TAT GTT GTG GGT TAT CAA GAC AAA TGG GAT  190
 48   I   D   V   T   D   V   Y   V   V   G   Y   Q   D   K   W   D   63

191  GGC AAA GAT CGA GCT GTT TTC CTT GAC AAG GTT CCT ACT GTT GCA ACT  238
 64   G   K   D   R   A   V   F   L   D   K   V   P   T   V   A   T   79

239  AGT AAA CTT TTC CCA GGG GTG ACT AAT CGT GTA ACG TTA ACA TTT GAT  286
 80   S   K   L   F   P   G   V   T   N   R   V   T   L   T   F   D   95

287  GGC AGC TAT CAG AAA CTT GTG AAT GCT GCG AAA GTG GAT AGA AAG GAT  334
 96   G   S   Y   Q   K   L   V   N   A   A   K   V   D   R   K   D  111

335  CTC GAA CTG GGC GTC TAC AAA CTC GAG TT                          363
112   L   E   L   G   V   Y   K   L   E                        120 (136)
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SEQ IN No. 9

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Tyr Asn Thr Val Ser Phe Asn Leu Gly Glu Ala Tyr Glu Tyr Pro Thr   16

Phe Ile Gln Asp Leu Arg Asn Glu Leu Ala Lys Gly Thr Pro Val Cys   32

Gln Leu Pro Val Thr Leu Gln Thr Ile Ala Asp Asp Lys Arg Phe Val   48

Leu Val Asp Ile Thr Thr Thr Ser Lys Lys Thr Val Lys Val Ala Ile   64

Asp Val Thr Asp Val Tyr Val Val Gly Tyr Gln Asp Lys Trp Asp Gly   80

Lys Asp Arg Ala Val Phe Leu Asp Lys Val Pro Thr Val Ala Thr Ser   96

Lys Leu Phe Pro Gly Val Thr Asn Arg Val Thr Leu Thr Phe Asp Gly  112

Ser Tyr Gln Lys Leu Val Asn Ala Ala Lys Val Asp Arg Lys Asp Leu  128

Glu Leu Gly Val Tyr Lys Leu Glu Phe Ser Ile Glu Ala Ile Trp Gly  144

Lys Thr Gln Asn Gly                                              149
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Note: Trp-143 is uncertain